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(54) Human insulin-like growth factor analogs with reduced binding to serum carrier proteins and their production in yeast.

(57) A synthetic gene encoding a 71-amino acid analog of human insulin-like growth factor (hIGF-I) has been constructed and expressed in the yeast, Saccharomyces cerevisiae. The protein analog, IGF132, contains the first 17 amino acids of the B chain of human insulin in place of the first 16 amino acids of hIGF-I. The purified hybrid protein has high affinity for the type I IGF receptor (12 nM) yet has drastically reduced affinity for human serum carrier proteins (>1000 nM). This analog is 5 to 10 times more active than normal hIGF-I in stimulating DNA synthesis in 3T3 cells and is a more active growth factor in vivo due to its reduced affinity for serum carrier proteins. Other proteins with similar properties have also been constructed. The protein analogs thus have a variety of utilities such as in promoting lactation in animals; promoting growth and feed efficiency in animals; improving carcass quality by increasing lean and decreasing fat; promoting wound healing in animals, including humans; promoting glucose utilization in skeletal muscle, and stimulating erythropoiesis, the production of red blood cells.

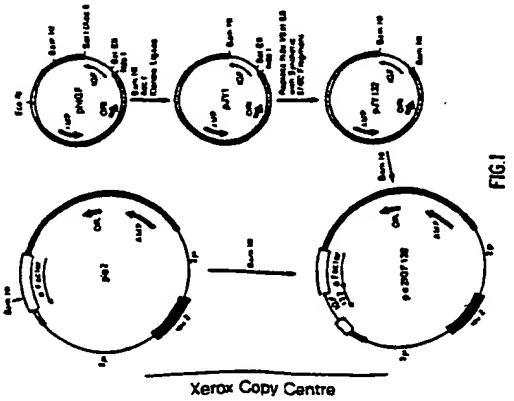

FIG.1

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HUMAN INSULIN-LIKE GROWTH FACTOR ANALOG WITH REDUCED BINDING TO SERUM CARRIER PROTEINS AND THEIR PRODUCTION IN YEAST

BACKGROUND OF THE INVENTION

The incorporation of fragments of the insulin molecule into IGF-I has previously been attempted in the form of two-chain disulfide-linked insulin-like structures. These molecules have considerably reduced biological activity relative to IGF-I and serum carrier protein binding is still significant rendering the in vitro activity of such compounds of little in vivo utility. See Joshi et al. Biochemistry 24: 4208-42 (1985); DeVroede et al. Proc. Nat. Acad. Sci. U.S.A. 82: 3010-14 (1985); and Joshi et al. Biochem. and Biophys. Res. Comm. 133: 423-429 (1985). The IGF-I analogs described in this invention are produced as single chain IGF-I-like molecules with equal potency to IGF-I at the type I IGF receptor and very little serum protein binding rendering such analogs of significant potential in vivo utility.

SUMMARY OF THE INVENTION

Human insulin-like growth factor I (hIGF-I. also called somatomedin C) is a 70-amino acid protein purified from human serum. It is believed to mediate many of the effects of growth hormone; in particular it has been demonstrated to stimulate growth in hypophysectomized rats. In addition. IGF-I has been shown to promote cell growth and differentiation of various cell types.

Human IGF-I shows a remarkable amino acid sequence homology to insulin. This homology is the basis of a computer generated three-dimensional structural model for hIGF-I. (Blundell et al. Proc. Natl. Acad. Sci. U.S.A. 75: 180-184 (1978) and Blundell et al. Fed. Proc. Am. Soc. Exp. Biol. 42: 2592-2597 (1983)). This model predicts that a portion of the insulin receptor binding region is conserved within the IGF-I molecule explaining the ability of hIGF-I to bind to insulin receptors. The model also suggests regions of hIGF-I molecule which may be responsible for binding to serum carrier proteins.

One of the major differences between hIGF-I and insulin is that in normal human blood. greater than 99% of the IGF-I is bound to serum carrier proteins which do not readily cross the capillary barrier. Thus most of the IGF in serum is inactive. The physiological significance of the IGF carrier protein complex is not clear. The presence of serum binding proteins is a barrier to the bioactivity and bioavailability of exogenously administered IGF-I.

Investigations into the role of serum binding proteins in the bioactivity of IGF-I could lead potentially to important bioactive compounds. Our approach was to create a IGF-I analog that retains efficient binding to the type I receptor, yet would have reduced binding to serum carrier proteins. The design of this analog is based on the observation that insulin does not bind to serum carrier proteins. Evidence from synthetic, insulin-like two chain analogs suggests that amino acids of IGF-I responsible for carrier protein binding are in the B region of IGF-I. Therefore a synthetic gene for human IGF-I was modified to encode an IGF-I analog in which the first 16 amino acids of hIGF-I are replaced by the first 17 amino acids of the B chain of human insulin. The synthetic gene is then placed in a yeast recombinant DNA expression system and the peptide analog which is produced by the modified yeast cells is extracted therefrom and purified. Additional modifications of the IGF-I molecule have been carried out leading to additional analogs, all of which have substantial IGF-I type I receptor binding and reduced binding to serum carrier proteins.

Thus. it is an object of this invention to describe the preparation of synthetic genes encoding for IGF-I analogs and to describe the incorporation of such genes in a microorganism. A further object is to describe the preparation of the IGF-I analogs from culturing the genetically modified micro-organism. A still further object of this invention is to describe the properties and uses of the IGF-I analogs thus prepared. Still further objects will become apparent from reading the following description.

DESCRIPTION OF THE INVENTION

We have expressed a synthetic gene ncoding a 71-amino acid analog f human IGF-I. This analog IGF132, contains the first 17 amino acids of human insulin B chain in place of the first 16 amino acids of hIGF-I. The analog has near equal affinity for the type I IGF receptor as compar d to normal human IGF-I (Figure 6). Analog IGF132. however, has greatly reduced binding t both human and rat serum carrier

proteins (Figure 7 and 8). Thus, this new protein retains nearly full activity at the type I IGF receptor but does not bind to serum components. It is expected that this analog will be more potent in vivo than normal IGF-I. This analog is 10 times more potent than normal IGF-I in stimulating DNA synthesis in 3T3 cells (Figure 9).

The synthetic genes of this invention encode for a peptide which is an analog of human insulin-like growth factor (hIGF-I) and has the following structure where the letter designation for the constituent amino acids have the definitions given below:

A_1-A_2-A_3-A_4-LCG-A_5-A_6-LV-A_7-AL-A_8-A_9-R

wherein:

A_1 is G, V, or FV;
A_2 is P or N;
A_3 is E or Q;
A_4 is T, H or A;
A_5 is A or S;
A_6 is E or H;
A_7 is D or E;
A_8 is Q or Y;
A_9 is F or L; and
R is the remainder of the hIGF-I peptide consisting of 54 amino acids as follows:
VCGDRGFYFNKPTGYGSSSRRAPQTGIV
DECCFRSCDLRRLEMYCAPLKPAKSA
with the exception that the following gene: GPETLCGAELVDALQF-R which is the wild type hIGF-I and is excluded from the foregoing definition.

While the amino acid letter designations are generally well known to those skilled in the art, for purposes of clarity, the definitions as used herein are as follows:

A - Alanine
C - Cysteine
D - Aspartic acid
E - Glutamic acid
F - Phenylalanine
G - Glycine
H - Histidine
I - Isoleucine
K - Lysine
L - Leucine
M - Methionine
N - Asparagine
P - Proline
Q - Glutamine
R - Arginine
S - Serine
T - Threonine
V - Valine
Y - Tyrosine

Preferred variations of the foregoing peptide analogs are as follows:

A_1 is G, V or FV;
A_2 is P or N;
A_3 is Q;
A_4 is A;
A_5 is A or S;
A_6 is E or H;
A_7 is D or E;
A_8 is Y; and

A_9 is L.

Additionally, specific examples of such compounds ar as follows:
FVNQHLCGSHLVEALYL-R (Compound A or IGF132)
GPETLCGAELVDALYL-R (Compound B or IGF122)
GPQALCGAELVDALQF-R (Compound C or IGF130)
GPQALCGAELVDALYL-R (Compound D or IGF252)
VNQHLCGSHLVGALYL-R

The peptide analogs can be produced by procedures similar to methods existing for the preparation of natural hIGF-I peptide, and modifications thereof which would be well-known to those skilled in the art. Specifically, these analogs may be synthesized chemically using procedures developed for human IGF-I. See for example Li et al. Proc. Natl. Acad. Sci. U.S.A. 80: 2216-2220 (1983). In accordance with the present invention the IGF-I analogs may also be produced following the transformation of susceptible bacterial, yeast or tissue culture cell hosts with recombinant plasmids that include DNA sequences capable of directing the expression of IGF-I analogs. The DNA sequence may be prepared synthetically, chromosomally, by recombinant DNA techniques or combination thereof. DNA sequences capable of directing the expression of IGF-I analogs could also be introduced into the germ line of animals or extra chromasomally to produce transgenic animals endogenously producing the IGF-I analogs.

The synthetic genes of this invention are prepared using recombinant DNA biotechnology techniques well known to those skilled in the art. Figure 1 outlines the steps in combining the plasmids pα2 and phIGF with the inclusion of the synthetic gene of this invention.

The instant synthetic gene produces analogs of hIGF-I which have substantial activity but, because they are not apparently bound to serum proteins have levels of activity which, when taken on a molar or weight basis are considerably more active than wild-type hIGF-I. The compounds are thus highly active as agents to increase the yield and efficency of milk production of animals, particularly ruminant animals such as cows. The compounds are also useful as growth promotant agents in food producing animals by increasing the rate of gain, feed efficency and carcass quality. The compounds are further useful as agents to promote wound healing and to stimulate erythropoiesis (the manufacture of red blood cells).

When used to increase milk production or as an animal growth promotant the compounds are administered parenterally such as by subcutaneous, intramuscular or intravenous injection or by a sustained release subcutaneous implant. In subcutaneous, intramuscular and intravenous injection the active ingredient is dissolved or dispersed in a liquid carrier vehicle. For parenteral administration, the active material is suitably admixed with an acceptable vehicle, preferably of the vegetable oil variety such as peanut oil, cotton seed oil and the like. Other parenteral vehicles such as organic preparation using solketal, glycerol, formal and aqueous parenteral formulations are also used. The active compound or compounds are dissolved or suspended in the parenteral formulation for administration; such formulations generally contain from 0.005 to 5% by weight of the active compound.

The instant compounds are effective by significantly increasing the level of milk production or the rate of weight gain or feed efficiency when administered at levels of from 0.1 to 100 mg per kg of animal body weight, preferably at from 1 to 10 mg/kg. When the compounds are administered in the form of a subcutaneous implant the compound is suspended or dissolved in a slowly dispersed material known to those skilled in the art, or administered in a device which slowly releases the active material through the use of a constant driving force such as an osmotic pump. In such cases constant administration over periods ranging from 20 to 120 days are possible with the active ingredient being released at from 0.1 to 10 mg.kg.day.

Because the hIGF-I analogs act synergistically with platelet-derived growth factor (PDGF) or other competence factors such as fibroblast growth factor (FGF) to stimulate DNA synthesis and cell replication in human fibroblasts, such analogs are useful to promote wound healing especially in cases where endogenous hIGF levels are low. Thus, the instant IGF-I analogs may be administered in combination with PDGF or FGF. The compounds could be administered parenterally, either subcutaneously, intramuscularly or intravenously using pharmaceutically acceptable parenteral formulation ingredients such as those listed above. The compounds would be administered at a dose of from 0.1 to 100 mg/kg, preferably from 1 to 10 mg.kg. Preferably, however, the compounds are administered topically when used as an agent to promote wound healing. Typical formulations for topical application are liquid, paste, ointment and spray formulations. The formulations could also be incorporated into a dressing which would be applied to the wound. The dressing would slowly release the compound directly to th site needing treatment.

The compounds would be incorporated into the topical formulation at concentrations of from 0.003 to 10% by weight with most formulations requiring from 0.3 to 3%. The concentration could be adjusted to

provide for daily doses of from 0.06 to 2 mg of the active compound with allowance made to provide for multiple applications during any particular day.

The instant compounds may also be useful as erythropoietic agents possibly by virtue of their ability to stimulate late erythroid precursor differentiation. In such cases the compounds are administered parenterally as described above. The compounds may be administered either alone or in combination with erythropoietin to promote the production of red blood cells. For such uses the compounds are administered at doses of from 0.1 to 100 mg/kg, preferably from 1 to 10 mg/kg. Such doses are on a daily basis and if needed, the dose may be divided into multiple daily doses.

Attached hereto are figures which further describe and explain the instant invention.

Figure 1 describes the preparation of the recombinant plasmid pα2IGF132 from plasmid pα2 and plasmid phIGF by selective cleavage and recombination. The plasmid encodes for the 71-amino acid analog of human IGF-I.

Figure 2A describes a replacement gene fragment for the NdeI/BstEII position of plasmid pJY1. The replacement fragment was in turn formed by the ligation of four oligonucleotides IGF132, IGF133, IGF134 and IGF135.

Figure 3A describes the DNA gene sequence and the analog it encodes which is inserted by ligation into plasmid pα2IGF132.

Figures 3B, 3C and 3D similarly describe the DNA gene sequence and analogs for IGF122, IGF130 and IGF252 respectively.

Figure 4 describes the elution profile of analog IGF132 in a Biogel P10 gel filtration column.

Figure 5 describes the purification of the Biogel P10 active peaks from the preparation of A (IGF132), B (IGF122), C (IGF130) and D (IGF252) by high pressure liquid chromatography.

Figure 6 describes the binding of analogs A (IGF132) B (IGF122), C (IGF130) and D (IGF252) to type I IGF receptors in comparison to wild type recombinant hIGF-I. In the figure, analog A is represented by "O", B by "◊", C by "◆" and D by "▲", and wild type IGF-I by "●".

Figure 7 describes the binding of analog A (IGF132) B (IGF122), C (IGF130) and D (IGF252) to human serum carrier proteins in comparison to the binding of wild type hIGF-I. The hIGF-I is tightly bound to serum carrier proteins while analogs IGF132 and IGF252 are very weakly bound. The same representations shown in Figure 6 are employed in this figure.

Figure 8 describes the binding of Analog A (IGF132) and hIGF-I to native binding protein in rat serum. The h-IGF-I binds in a saturable fashion whereas binding of analog A (IGF132) is not observed.

Figure 9 describes a comparison of biological activities of IGF-I with analogs A (IGF132) and D (IGF252) in the ability to stimulate DNA synthesis in 3T3 cells. Analogs A and D are observed to be 10 times more potent than wild-type IGF-I.

Figure 10 describes a comparison of the ability of IGF-I and IGF252 to stimulate glycogen synthesis in rat diaphragm (part A) or lipid synthesis in rat adipose tissue (part B) in vivo. IGF252 is at least 2 fold more potent than IGF-I in stimulating glycogen synthesis in vivo. Neither IGF-I nor IGF252 stimulate lipid synthesis at these doses.

EXAMPLE

Construction of the IGF132 Analog Gene

A synthetic gene encoding the 70 amino acids of hIGF-I has been assembled and cloned into pBR322 to yield plasmid phIGF. Plasmid phIGF was modified to form plasmid pJY1 as described in Figure 1. Four oligonucleotides: IGF132-5′ TATG CCGC ATC CTT TCC TTG GAT AAA AGA TTT GTA AAC CAA CAT 3′; IGF133-5′ ACA CAA ATG TTG GTT TAC AAA TCT TTT ATC CAA GGA AAG GAT CCG GCA 3′; IGF134-5′ TTG TGT GGC TCC CAT CTG GTT GAA GCT TTG TAC TTG GTT TGC G 3′; and IGF135-5′ GTC ACC GCA AAC CAA GTA CAA AGC TTC AAC GAG ATG GGA GCC 3′ were ligated to form a NdeI/BstEII replacement fragment (Figure 2). This fragment was inserted into pJY1 digested with endonuclease NdeI and BstEII. Transformation of E. coli with the ligation mixture yields bacteria carrying the plasmid pJY132. The DNA sequence and the analog IGF it encodes is shown in Figure 3A.

Expression of Analog IGF132

The Bam HI IGF132 gene cassette from plasmid pJY132 was ligated into Bam HI digested pα2 as indicated in Figure 1. The plasmid with the IGF132 cassette in pα2 in the prop r orientation was designated pα2IGF132. This plasmid was introduced into the yeast strain BJ1995. Yeast strain carrying the pα2IGF132 plasmid secrete the protein IGF132 into the growth media.

Expression and Purification of Mutant hIGF I Peptides

Saccharomyces cerevisiae strain BJ1995 (MAT α, leu2, trpl, ura3, prbl-1122, pep4-3, cir) was transformed with the appropriate expression plasmid and transformants were selected on leucine minus plates. Cells were grown to saturation in 1 liter of 5x leu(-) media, pH 4.8, containing 0.85% yeast nitrogen base without amino acids and ammonium sulfate supplemented with 4% glucose, 1% ammonium sulfate, 0.6% sodium hydroxide, 0.03% L-isoleucine, 0.03% L-phenylalanine, 0.025% L-tyrosine, 0.02% L-lysine, 0.02% L-tryptophan, 0.02% uracil, 0.02% adenine, 0.01% L-arginine, 0.005% methionine, 0.005% L-histidine, 29 µM ferric chloride, 25 µM zinc sulfate, and 1% succinic acid. Cells were removed by centrifugation at 3000 x g. The cleared supernatant was mixed with 10 g of BioRex 70 equilibrated in 1% succinic acid, pH 4.8. After stirring for 3 hours at 4° C, the resin was poured into a 2.5 cm column and washed with 1L of 1% succinic acid, pH 4.8. The peptide was eluted with 1M ammonium acetate, pH 8. Receptor active material was pooled, concentrated to 4 ml, then applied to a 2.5 x 90 cm Biogel P10 (200-400 mesh) column equilibrated in 1N acetic acid. Gel filtration was carried out at 30 ml per hour. Twelve ml fractions were collected and assayed for IGF-like activity by the radioreceptor assay. Active fractions were pooled and lyophilized. The activity was reconstituted in 0.2 ml 0.05% trifluoroacetic acid, 15% acetonitrile and loaded onto a C18 µBondapak (0.46 x 25 cm, 10 micron, Waters) reverse phase HPLC column. The peptides were eluted from the column using a 15-50% acetonitrile gradient in 0.05% trifluoroacetic acid. The flow rate was 1 ml per minute and 1 minute fractions were collected and assayed by receptor assay. Active fractions were pooled and lyophilized. The purified peptide was quantitated by amino acid analysis and stored at -20° C in 0.1 N acetic acid at a concentration of 0.1 mM.

Characterization of IGF Analogs

Quantitative amino acid analysis was employed to determine the concentration of purified analogs. The amino acid composition is consistent with that expected for the analogs.

Binding of the analogs to type I IGF receptor is shown in Figure 6. Analog A (IGF132), B (IGF122), C (IGF130) and D (IGF252) inhibit the binding of ^{125}I-hIGF-I to human placental membranes with a IC_{50} of 12 nM, 4.5 nM, 5.3 nM, and 5.0 nM respectively, compared to 5.6 nM for wild type recombinant hIGF-I. Binding of analog 132 to human serum carrier proteins is shown in Figure 7. Recombinant wild type hIGF-I inhibits binding of ^{125}I-hIGF-I to acid stable human carrier proteins with a IC_{50} of 0.42 nM, analog IGF132 showed little ability to inhibit this binding with a IC_{50} > 1000 nM. IGF130, IGF122 and IGF252 inhibit binding with IC_{50} values of 1.8 nM, 2.1 nM and 300 nM respectively.

^{125}I-labelled analog IGF132 was monitored for the ability to bind components in normal rat serum. When ^{125}I-IGF or ^{125}I-IGF132 is chromatographed without prior incubation with serum, the radioactivity is eluted in a broad peak which migrates at the position expected for a 7.5 kD peptide (Figure 8A). After incubation of ^{125}I-IGF with rat serum, a radioactive peak appears which elutes at the position expected for a 150 kD protein, and the amount of radioactivity in the free ^{125}I-αIGF peak decreases (Figure 8B (●)). The ^{125}I-αIGF bound to the 150 kD species represents 36% ± 5% of the total ^{125}I-IGF-I in the incubation. When the incubation is performed in the presence of 1 µg unlabelled IGF, only one radioactive peak is observed and this corresponds to unbound ^{125}I-IGF (Figure 8 (0)). Thus, under the conditions of this assay, the binding of ^{125}I-IGF to the 150 kD species from rat serum is saturable.

After incubation of ^{125}I-IGF132 with rat serum, only free radioactive peptid is luted (Figure 8C ( )). The presence of 1 µg unlabelled IGF132 in the incubation does not significantly change the radioactive profile (Figure 8C (0)).

IGF-I stimulates DNA synthesis in mouse 3T3 cells. As shown in Figure 9, IGF252 and IGF132 stimulate

DNA synthesis in these cells with about 10-fold higher potency then wild type IGF-I.

IGF-I stimulates the incorporation of ^{14}C-glucose into glycogen in the rat diaphragm in vivo. This process is mediated by the type 1 IGF receptor. As shown in Figure 10, part A, IGF252 is at least two fold more potent than wild type IGF-I. As expected, neither IGF-I nor IGF252 stimulates the incorporation of ^{14}C-glucose into lipid in adipose tissue. Adipose tissue does not have type 1 IGF receptors.

Claims

1. A synthetic polypeptide analog of hIGF-I which has the structure:

A_1-A_2-A_3-A_4-LCG-A_5-A_6-LV-A_7-AL-A_8-A_9-R

wherein:
A_1 is G, V, or FV;
A_2 is P or N;
A_3 is E or Q;
A_4 is T, H or A;
A_5 is A or S;
A_6 is E or H;
A_7 is D or E;
A_8 is Q or Y;
A_9 is F or L; and
R is the remainder of the hIGF-I peptide, provided that and A_1 to A_9 groups and the other amino acids do not constitute GPETLCGAELVDALQF-R.

2. The peptide of Claim 1 wherein:
A_1 is G, V, or FV;
A_2 is P or N;
A_3 is Q;
A_4 is A;
A_5 is A or S;
A_6 is E or H;
A_7 is D or E;
A_8 is Y; and
A_9 is L.

3. The peptide of Claim 1 which is:
FVNQHLCGSHLVEALYL-R.

4. The peptide of Claim 1 which is:
GPETLCGAELVDALYL-R.

5. The peptide of Claim 1 which is:
GPQALCGAELVDALQF-R.

6. The peptide of Claim 1 which is:
GPQALCGAELVDALYL-R.

7. The peptide of Claim 1 which is:
VNQHLCGSHLVEALYL-R.

8. A synthetic gene encoding for the polypeptide of Claim 1.

9. A synthetic gene encoding for the polypeptide of Claim 3.

10. The synthetic gene of Claim 9 which is:

TATG CCGG ATC CTT TCC TTG GAT AAA AGA TTT GTA AAC CAA
AC GGCC TAG GAA AGG AAC CTA TTT TCT AAA CAT TTG GTT

CAT TTG TGT GGC TCC CAT CTC GTT GAA GCT TTG TAC TTG
GTA AAC ACA CCG AGG GTA GAG CAA CTT CGA AAC ATG AAC

GTT TGC GGT GAC CGC GGT TTC TAC TTC AAC AAA CCG ACT
CAA ACG CCA CTG GCG CCA AAG ATG AAG TTG TTT GGC TGA

GGT TAC GGT TCT TCT TCT AGA CGT GCT CCG CAG ACT GGT
CCA ATG CCA AGA AGA AGA TCT GCA CGA GGC GTC TGA CCA

ATC GTT GAT GAA TGC TGC TTC AGA TCT TGT GAC CTG CGT
TAG CAA CTA CTT ACG ACG AAG TCT AGA ACA CTG GAC GCA

CGT CTC GAG ATG TAC TGC GCA CCG CTG AAA CCG GCT AAA
GCA GAG CTC TAC ATG ACG CGT GGC GAC TTT GGC CGA TTT

TCT GCT TGA TAA GTCG
AGA CGA ACT ATT CAGCC TAG

11. A synthetic gene encoding for the polypeptide of Claim 4.

12. The synthetic gene of Claim 11 which is:

ATC CTT TCC TTG GAT AAA AGA GGT CCG GAA ACT TTG TGT
TAG GAA AGG AAC CTA TTT TCT CCA GGC CTT TGA AAC ACA
GGT GCT GAG CTC GTT GAC GCT CTG TAC CTC GTT TGC
CCA CGA CTC GAG CAA CTG CGA GAC ATG GAG CAA ACG

GGT GAC CGC GGT TTC TAC TTC AAC AAA CCG ACT GGT TAC
CCA CTG GCG CCA AAG ATG AAG TTG TTT GGC TGA CCA ATG
GGT TCT TCT TCT AGA CGT GCT CCG CAG ACT GGT ATC GTT
CCA AGA AGA AGA TCT GCA CGA GGC GTC TGA CCA TAG CAA

GAT GAA TGC TGC TTC AGA TCT TGT GAC CTG CGT CGT
CTA CTT ACG ACG AAG TCT AGA AGA CTG GAC GCA GCA

CTC GAG ATG TAC TGC GCA CCG CTG AAA CCG GCT AAA TCT
GAG CTC TAC ATG ACG CGT GGC GAC TTT GGC CGA TTT AGA
GCT TGA TAA GTCG
CGA ACT ATT CAGCCTAG

13. A synthetic gene encoding for the polypeptide of Claim 5.

14. The synthetic gene of Claim 13 which is:

ATC CTT TCC TTG GAT AAA AGA GGT CCG CAA GCT TTG TGT
TAG GAA AGG AAC CTA TTT TCT CCA GGC GTT CGA AAC ACA
GGT GCT GAG CTC GTT GAC GCT CTG CAG TTC GTT TGC
CCA CGA CTC GAG CAA CTG CGA GAC GTC AAG CAA ACG

GGT GAC CGC GGT TTC TAC TTC AAC AAA CCG ACT GGT TAC
CCA CTG GCG CCA AAG ATG AAG TTG TTT GGC TGA CCA ATG
GGT TCT TCT TCT AGA CGT GCT CCG CAG ACT GGT ATC GTT
CCA AGA AGA AGA TCT GCA CGA GGC CTC TGA CCA TAG CAA

GAT GAA TGC TGC TTC AGA TCT TGT GAC CTG CGT CGT
CTA CTT ACG ACG AAG TCT AGA AGA CTG GAC GCA GCA
CTC GAG ATG TAC TGC GCA CCG CTG AAA CCG GCT AAA TCT
GAG CTC TAC ATG ACG CGT GGC GAC TTT GGC CGA TTT AGA
GCT TGA TAA CTCG
CGA ACT ATT GAGCCTAG

15. A synthetic gene encoding for the polypeptide of Claim 6.

16. The synthetic gen of Claim 14 which is:

ATC CTT TCC TTG GAT AAA AGA GGT CCG CAA GCT TTG TGT
TAG GAA AGG AAC CTA TTT TCT CCA GGC GTT CGA AAC ACA

GGT GCT GAG CTC GTT GAC GCT CTG TAC CTC GTT TGC
CCA CGA CTC GAG CAA CTG CGA GAC ATG GAG CAA ACG

GGT GAC CGC GGT TTC TAC TTC AAC AAA CCG ACT GGT TAC
CCA CTG GCG CCA AAG ATG AAG TTG TTT GGC TGA CCA ATG
GGT TCT TCT TCT AGA CGT GCT CCG CAG ACT GGT ATC GTT
CCA AGA AGA AGA TCT GCA CGA GGC GTC TGA CCA TAG CAA

GAT GAA TGC TGC TTC AGA TCT TGT GAC CTG CGT CGT
CTA CTT ACG ACG AAG TCT AGA AGA CTG GAC GCA GCA

CTC GAG ATG TAC TGC GCA CCG CTG AAA CCG GCT AAA TCT
GAG CTC TAC ATG ACG CGT GGC GAC TTT GGC CGA TTT AGA
GCT TGA TAA GTCG
CGA ACT ATT CAGCCTAG

17. A process for the preparation of the synthetic gene of Claim 7 which comprises:
a) the synthesis of the appropriate constituent oligonucleotides;
b) annealing and ligation of said oligonucleotides to gene fragments; and
c) cloning of synthetic gene into recombinant DNA plasmid.

18. A process for the preparation of the polypeptide analog of Claim 1 by the recombinant DNA expression systems of bacteria, yeast or tissue culture cell hosts which comprises:
a) insertion of the appropriate synthetic gene into an expression vector to form an expression cassette;
b) introduction of the expression cassette into the bacteria, yeast or tissue cell culture host;
c) growth of the transformed expression host; and
d) purification of the desired polypeptide analog from said host.

19. A method for the promotion of lactation in animals which comprises administering to a lactating animal a synthetic polypeptide analog of hIGF-I of Claim 1.

20. A composition useful for the promotion of lactation in animals which comprises an inert carrier and a synthetic polypeptide analog of hIGF-I of Claim 1.

21. A method for promoting growth and feed efficency in animals which comprises administering to such animals a synthetic polypeptide analog of hIGF-I of Claim 1.

22. A composition useful for promoting growth and feed efficiency in animals which comprises an inert carrier and a synthetic polypeptide analog of hIGF-I of Claim 1.

23. A method for increasing the lean and decreasing the fat content of meat producing animals which comprises administering to such animals a synthetic polypeptide analog of hIGF-I of Claim 1.

24. A composition useful for increasing the lean and decreasing the fat content of meat producing animals which comprises an inert carrier and a synthetic polypeptide analo of hIGF-I of Claim 1.

25. The use of a synthetic polypeptide analog of hIGF-I of Claim 1, for the preparation of a medicament useful for promoting wound healing in animals.

26. A composition useful for promoting wound healing in animals which comprises an inert carrier and a synthetic polypeptide analog of hIGF-I of Claim 1.

27. A method for stimulating erythropoiesis in animals which comprises administering to an animal in need of erythropoiesis a synthetic polypeptide analog of hIGF-I of Claim 1.

28. A composition useful for stimulating erythropoiesis in animals which comprises an inert carrier and a synthetic polypeptide analog of hIGF-I of Claim 1.

Claims for the following contracting States: ES, GR

1.- A process for the preparation of the synthetic polypeptide analog of hIGF-I by the recombinant DNA expression systems of bacteria, yeast or tissue culture cells hosts, said polypeptide analog having the following structure:

A_1-A_2-A_3-A_4-LCG-A_5-A_6-LV-A_7-AL-A_8-A_9-R

wherein:
A_1 is G, V, or FV;
A_2 is P or N;
A_3 is E or Q;
A_4 is T, H or A;
A_5 is A or S;
A_6 is E or H;
A_7 is D or E;
A_8 is Q or Y;
A_9 is F or L; and
R is the remainder of the hIGF-I peptide, provided that and A_1 to A_9 groups and the other amino acids do not constitute GPETLCGAELVDALQF-R,
which comprises:

a) insertion of the appropriate synthetic gene into an expression vector to form an expression cassette;

b) introduction of the expression cassette into the bacteria, yeast or tissue cell culture host;

c) growth of the transformed expression host; and

d) purification of the desired polypeptide analog from said host.

2.- The process of claim 1, wherein
A_1 is G, V, or FV;
A_2 is P or N;
A_3 is Q;
A_4 is A;
A_5 is A or S;
A_6 is E or H;
A_7 is D or E;
A_8 is Y; and
A_9 is L.

3.- The process of claim 1, wherein the polypeptide obtained is FVNQHLCGSHLVEALYL-R.

4.- The process of claim 1, wherein the polypeptide obtained is GPETLCGAELVDALYL-R.

5.- The process of claim 1, wherein the polypeptide obtained is GPQALCGAELVDALQF-R.

6.- The process of claim 1, wherein the polypeptide obtained is GPQALCGAELVDALYL-R.

7.- The process of claim 1, wherein the polypeptide obtain-is VNQHLCGSHLVEALYL-R.

8.- A process for the preparation of a synthetic gene encoding for the polypeptide obtained in claim 1, which comprises:

a) the synthesis of the appropriate constituent oligonucleotides;

b) annealing and ligation of said oligonucleotides to gene fragments; and

c) cloning of synthetic gene into recombinant DNA plasmid.

9.- A process according to claim 8 for the preparation of the synthetic gene encoding for the polypeptide obtained in claim 3.

10.- The process of claim 9, wherein the synthetic gene obtained is:

TATG CCGG ATC CTT TCC TTG GAT AAA AGA TTT GTA AAC CAA
AC GGCC TAG GAA AGG AAC CTA TTT TCT AAA CAT TTG GTT

CAT TTG TGT GGC TCC CAT CTC GTT GAA GCT TTG TAC TTG
GTA AAC ACA CCG AGG GTA GAG CAA CTT CGA AAC ATG AAC

GTT TGC GGT GAC CGC GGT TTC TAC TTC AAC AAA CCG ACT
CAA ACG CCA CTG GCG CCA AAG ATG AAG TTG TTT GGC TGA

GGT TAC GGT TCT TCT TCT AGA CGT GCT CCG CAG ACT GGT

CCA ATG CCA AGA AGA AGA TCT GCA CGA GGC GTC TGA CCA

ATC GTT GAT GAA TGC TGC TTC AGA TCT TGT GAC CTG CGT
TAG CAA CTA CTT ACG ACG AAG TCT AGA ACA CTG GAC GCA

CGT CTC GAG ATG TAC TGC GCA CCG CTG AAA CCG GCT AAA
GCA GAG CTC TAC ATG ACG CGT GGC GAC TTT GGC CGA TTT

TCT GCT TGA TAA GTCG
AGA CGA ACT ATT CAGCC TAG

11.- A process according to claim 8 for the preparation of the synthetic gene encoding for the polypeptide obtained in claim 4.

12.- The process of claim 11, wherein the synthetic gene obtained is:

ATC CTT TCC TTG GAT AAA AGA GGT CCG GAA ACT TTG TGT
TAG GAA AGG AAC CTA TTT TCT CCA GGC CTT TGA AAC ACA
GGT GCT GAG CTC GTT GAC GCT CTG TAC CTC GTT TGC
CCA CGA CTC GAG CAA CTG CGA GAC ATG GAG CAA ACG

GGT GAC CGC GGT TTC TAC TTC AAC AAA CCG ACT GGT TAC
CCA CTG GCG CCA AAG ATG AAG TTG TTT GGC TGA CCA ATG
GGT TCT TCT TCT AGA CGT GCT CCG CAG ACT GGT ATC GTT
CCA AGA AGA AGA TCT GCA CGA GGC GTC TGA CCA TAG CAA

GAT GAA TGC TGC TTC AGA TCT TGT GAC CTG CGT CGT
CTA CTT ACG ACG AAG TCT AGA AGA CTG GAC GCA GCA

CTC GAG ATG TAC TGC GCA CCG CTG AAA CCG GCT AAA TCT
GAG CTC TAC ATG ACG CGT GGC GAC TTT GGC CGA TTT AGA
GCT TGA TAA GTCG
CGA ACT ATT CAGCCTAG

13.- A process according to claim 8, for the preparation of the synthetic gene encoding for the polypeptide obtained in claim 5.

14.- The process of claim 13, wherein the synthetic gene obtained is:

ATC CTT TCC TTG GAT AAA AGA GGT CCG CAA GCT TTG TGT
TAG GAA AGG AAC CTA TTT TCT CCA GGC GTT CGA AAC ACA
GGT GCT GAG CTC GTT GAC GCT CTG CAG TTC GTT TGC
CCA CGA CTC GAG CAA CTG CGA GAC GTC AAG CAA ACG

GGT GAC CGC GGT TTC TAC TTC AAC AAA CCG ACT GGT TAC
CCA CTG GCG CCA AAG ATG AAG TTG TTT GGC TGA CCA ATG
GGT TCT TCT TCT AGA CGT GCT CCG CAG ACT GGT ATC GTT
CCA AGA AGA AGA TCT GCA CGA GGC CTC TGA CCA TAG CAA

GAT GAA TGC TGC TTC AGA TCT TGT GAC CTG CGT CGT
CTA CTT ACG ACG AAG TCT AGA AGA CTG GAC GCA GCA
CTC GAG ATG TAC TGC GCA CCG CTG AAA CCG GCT AAA TCT
GAG CTC TAC ATG ACG CGT GGC GAC TTT GGC CGA TTT AGA
GCT TGA TAA CTCG
CGA ACT ATT GAGCCTAG

15. A process according to claim 8, for the preparation of the synthetic gene encoding for the polypeptide obtained in claim 6.

16.- The process of claim 15, wherein the synth tic gene obtained is:

ATC CTT TCC TTG GAT AAA AGA GGT CCG CAA GCT TTG TGT
TAG GAA AGG AAC CTA TTT TCT CCA GGC GTT CGA AAC ACA
GGT GCT GAG CTC GTT GAC GCT CTG TAC CTC GTT TGC

CCA CGA CTC GAG CAA CTG CGA GAC ATG GAG CAA ACG

GGT GAC CGC GGT TTC TAC TTC AAC AAA CCG ACT GGT TAC
CCA CTG GCG CCA AAG ATG AAG TTG TTT GGC TGA CCA ATG
GGT TCT TCT TCT AGA CGT GCT CCG CAG ACT GGT ATC GTT
CCA AGA AGA AGA TCT GCA CGA GGC GTC TGA CCA TAG CAA

GAT GAA TGC TGC TTC AGA TCT TGT GAC CTG CGT CGT
CTA CTT ACG ACG AAG TCT AGA AGA CTG GAC GCA GCA

CTC GAG ATG TAC TGC GCA CCG CTG AAA CCG GCT AAA TCT
GAG CTC TAC ATG ACG CGT GGC GAC TTT GGC CGA TTT AGA
GCT TGA TAA GTCG
CGA ACT ATT CAGCCTAG.

17.- A method for the promotion of lactation in animals which comprises administering to a lactating animal the synthetic polypeptide analog of hIGF-I obtained through the process of claim 1.

18.- A method for promoting growth and feed efficiency in animals which comprises administering to such animals the synthetic polypeptide analog of hIGF-I obtained through the process of claim 1.

19.- A method of increasing the lean and decreasing the fat content of meat producing animals which comprises adminstering to such animals the synthetic polypeptide analog of hIGF-I obtained through the process of claim 1.

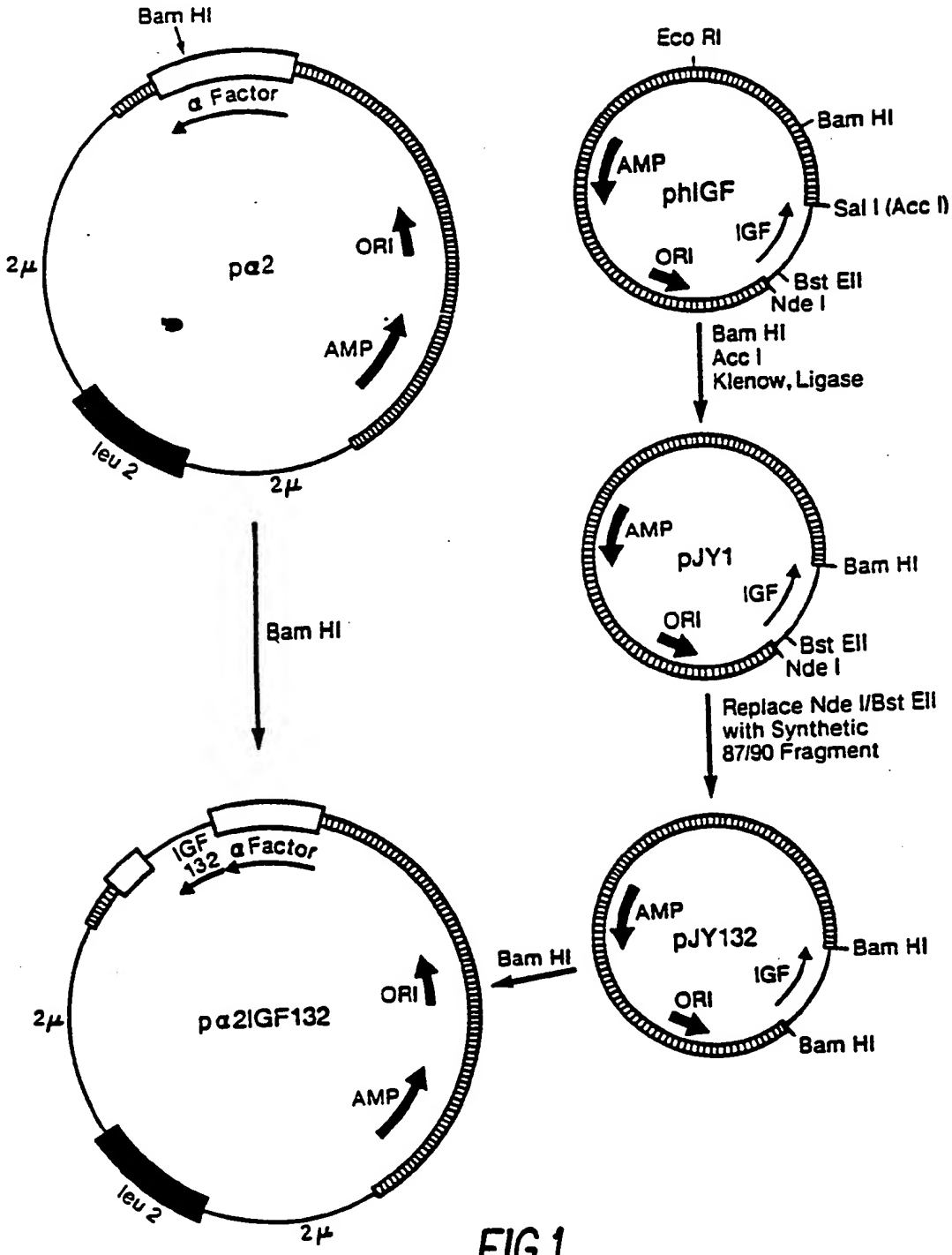

FIG.1

Nde I

Ile Leu Ser Leu Asp Lys Arg Phe Val Asn Gln His

TATGCCGG ATC CTT TCC TTG GAT AAA AGA TTT GTA AAC CAA CAT

ACGGCC TAG GAA AGG AAC CTA TTT TCT AAA CAT TTG GTT GTA AAC ACA

BstE II

Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys

TTG TGT GGC TCC CAT CTG GTT GAA GCT TTG TAC TTG GTT TGC G

CCG AGG GTA GAC CAA CTT CGA AAC ATG AAC CAA ACG C CACTG

FIG.2

```
                                                    ATC CTT TCC TTG GAT AAA AGA
                                                    TAG GAA AGG AAC CTA TTT TCT

Phe Val Asn Gln His Leu Cys Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Cys
TTT GTA AAC CAA CAT TTG TGT GGC TCC CAT CTC GTT GAA GCT TTG TAC TTG GTT TGC
AAA CAT TTG GTT GTA AAC ACA CCG AGG GTA GAG CAA CTT CGA AAC ATG AAC CAA ACG

Gly Asp Arg Gly Phe Tyr Phe Asn Lys Pro Thr Gly Tyr Gly Ser Ser Ser Arg Arg
GGT GAC CGC GGT TTC TAC TTC AAC AAA CCG ACT GGT TAC GGT TCT TCT TCT AGA CGT
CCA CTG GCG CCA AAG ATG AAG TTG TTT GGC TGA CCA ATG CCA AGA AGA AGA TCT GCA

Ala Pro Gln Thr Gly Ile Val Asp Glu Cys Cys Phe Arg Ser Cys Asp Leu Arg Arg
GCT CCG CAG ACT GGT ATC GTT GAT GAA TGC TGC TTC AGA TCT TGT GAC CTG CGT CGT
CGA GGC GTC TGA CCA TAG CAA CTA CTT ACG ACG AAG TCT AGA ACA CTG GAC GCA GCA

Leu Glu Met Tyr Cys Ala Pro Leu Lys Pro Ala Lys Ser Ala  *  *
CTC GAG ATG TAC TGC GCA CCG CTG AAA CCG GCT AAA TCT GCT TGA TAA GTCG
GAG CTC TAC ATG ACG CGT GGC GAC TTT GGC CGA TTT AGA CGA ACT ATT CAGCC TAG
```

FIG.3A

IGF-122

Gly Pro Glu Thr Leu Cys Gly Ala Glu Leu Val Asp Ala Leu Tyr Leu Val Cys
ATC CTT TCC TTG GAT AAA AGA GGT CCG GAA ACT TTG TGT GGT GCT GAG CTC GTT GAC GCT CTG TAC CTC GTT TGC
TAG GAA AGG AAC CTA TTT TCT CCA GGC CTT TGA AAC ACA CCA CGA CTC GAG CAA CTG CGA GAC ATG GAG CAA ACG

Gly Asp Arg Gly Phe Tyr Phe Asn Lys Pro Thr Gly Tyr Gly Ser Ser Ser Arg Arg
GGT GAC CGC GGT TTC TAC TTC AAC AAA CCG ACT GGT TAC GGT TCT TCT TCT AGA CGT
CCA CTG GCG CCA AAG ATG AAG TTG TTT GGC TGA CCA ATG CCA AGA AGA AGA TCT GCA

Ala Pro Gln Thr Gly Ile Val Asp Glu Cys Cys Phe Arg Ser Cys Asp Leu Arg Arg
GCT CCG CAG ACT GGT ATC GTT GAT GAA TGC TGC TTC AGA TCT TGT GAC CTG CGT CGT
CGA GGC GTC TGA CCA TAG CAA CTA CTT ACG ACG AAG TCT AGA AGA CTG GAC GCA GCA

Leu Gly Met Tyr Cys Ala Pro Leu Lys Pro Ala Lys Ser Ala * *
CTC GAG ATG TAC TGC GCA CCG CTG AAA CCG GCT AAA TCT GCT TGA TAA GTCG
GAG CTC TAC ATG ACG CGT GGC GAC TTT GGC CGA TTT AGA CGA ACT ATT CAGCCTAG

FIG. 3B

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                                Gly Pro Gln Ala Leu Cys Gly Ala Glu Leu Val Asp Ala Leu Gln Phe Val Cys
ATC CTT TCC TTG GAT AAA AGA GGT CCG CAA GCT TTG TGT GGT GCT GAG CTC GTT GAC GCT CTG CAG TTC GTT TGC
TAG GAA AGG AAC CTA TTT TCT CCA GGC GTT CGA AAC ACA CCA CGA CTC GAG CAA CTG CGA GAC GTC AAG CAA ACG

Gly Asp Arg Gly Phe Tyr Phe Asn Lys Pro Thr Gly Tyr Gly Ser Ser Ser Arg Arg
GGT GAC CGC GGT TTC TAC TTC AAC AAA CCG ACT GGT TAC GGT TCT TCT TCT AGA CGT
CCA CTG GCG CCA AAG ATG AAG TTG TTT GGC TGA CCA ATG CCA AGA AGA AGA TCT GCA

Ala Pro Gln Thr Gly Ile Val Asp Glu Cys Cys Phe Arg Ser Cys Asp Leu Arg Arg
GCT CCG CAG ACT GGT ATC GTT GAT GAA TGC TGC TTC AGA TCT TGT GAC CTG CGT CGT
CGA GGC GTC TGA CCA TAG CAA CTA CTT ACG ACG AAG TCT AGA AGA CTG GAC GCA GCA

Leu Glu Met Tyr Cys Ala Pro Leu Lys Pro Ala Lys Ser Ala  *   *
CTC GAG ATG TAC TGC GCA CCG CTG AAA CCG GCT AAA TCT GCT TGA TAA GTCG
GAG CTC TAC ATG ACG CGT GGC GAC TTT GGC CGA TTT AGA CGA ACT ATT CAGCCTAG
```

FIG.3C

IGF-252

Gly Pro Gln Ala Leu Cys Gly Ala Glu Leu Val Asp Ala Leu Tyr Leu Val Cys
ATC CTT TCC TTG GAT AAA AGA GGT CCG CAA GCT TTC TGT GGT GCT GAG CTC GTT GAC GCT CTG TAC CTC GTT TGC
TAG GAA AGG AAC CTA TTT TCT CCA GGC GTT CGA AAC ACA CCA CGA CTC GAG CAA CTG CGA GAC ATG GAG CAA ACG

Gly Asp Arg Gly Phe Tyr Phe Asn Lys Pro Thr Gly Tyr Gly Ser Ser Ser Arg Arg
GGT GAC CGC GGT TTC TAC TTC AAC AAA CCG ACT GGT TAC GGT TCT TCT TCT AGA CGT
CCA CTG GCG CCA AAG ATG AAG TTG TTT GGC TGA CCA ATG CCA AGA AGA AGA TCT GCA

Ala Pro Gln Thr Gly Ile Val Asp Glu Cys Cys Phe Arg Ser Cys Asp Leu Arg Arg
GCT CCG CAG ACT GGT ATC GTT GAT GAA TGC TGC TTC AGA TCT TGT GAC CTG CGT CGT
CGA GGC GTC TGA CCA TAG CAA CTA CTT ACG ACG AAG TCT AGA AGA CTG GAC GCA GCA

Leu Glu Met Tyr Cys Ala Pro Leu Lys Pro Ala Lys Ser Ala * *
CTC GAG ATG TAC TGC GCA CCG CTG AAA CCG GCT AAA TCT GCT TGA TAA GTCG
GAG CTC TAC ATG ACG CGT GGC GAC TTT GGC CGA TTT AGA CGA ACT ATT CAGCCTAG

FIG. 3D

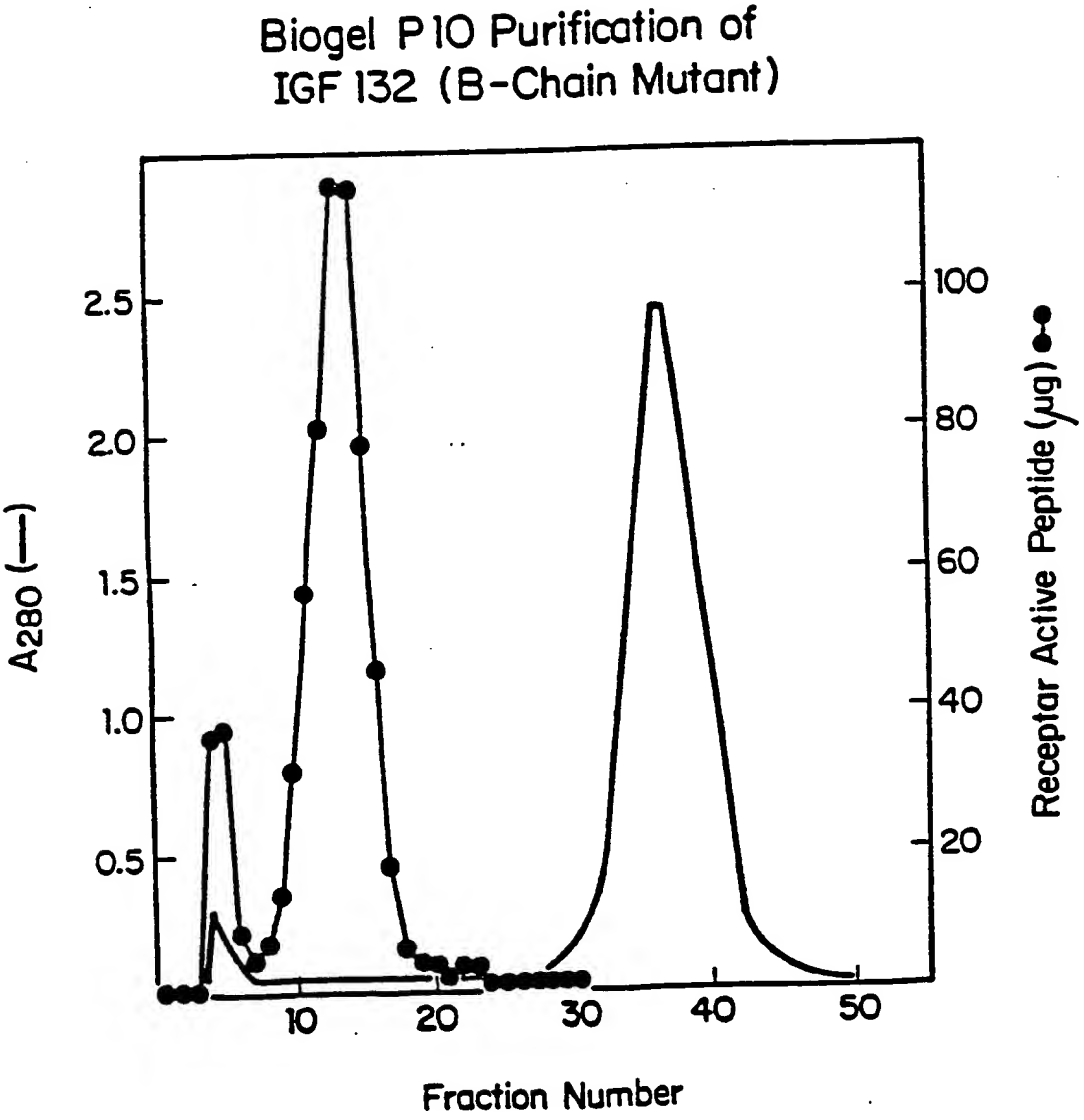


FIG.4

HPLC Purification of B-Chain Mutant (A), [Tyr 15, Leu 16] IGF I (B), [Gln 3, Ala 4] IGF I (C) and [Gln 3, Ala 4, Tyr 15, Leu 16] IGF I (D)

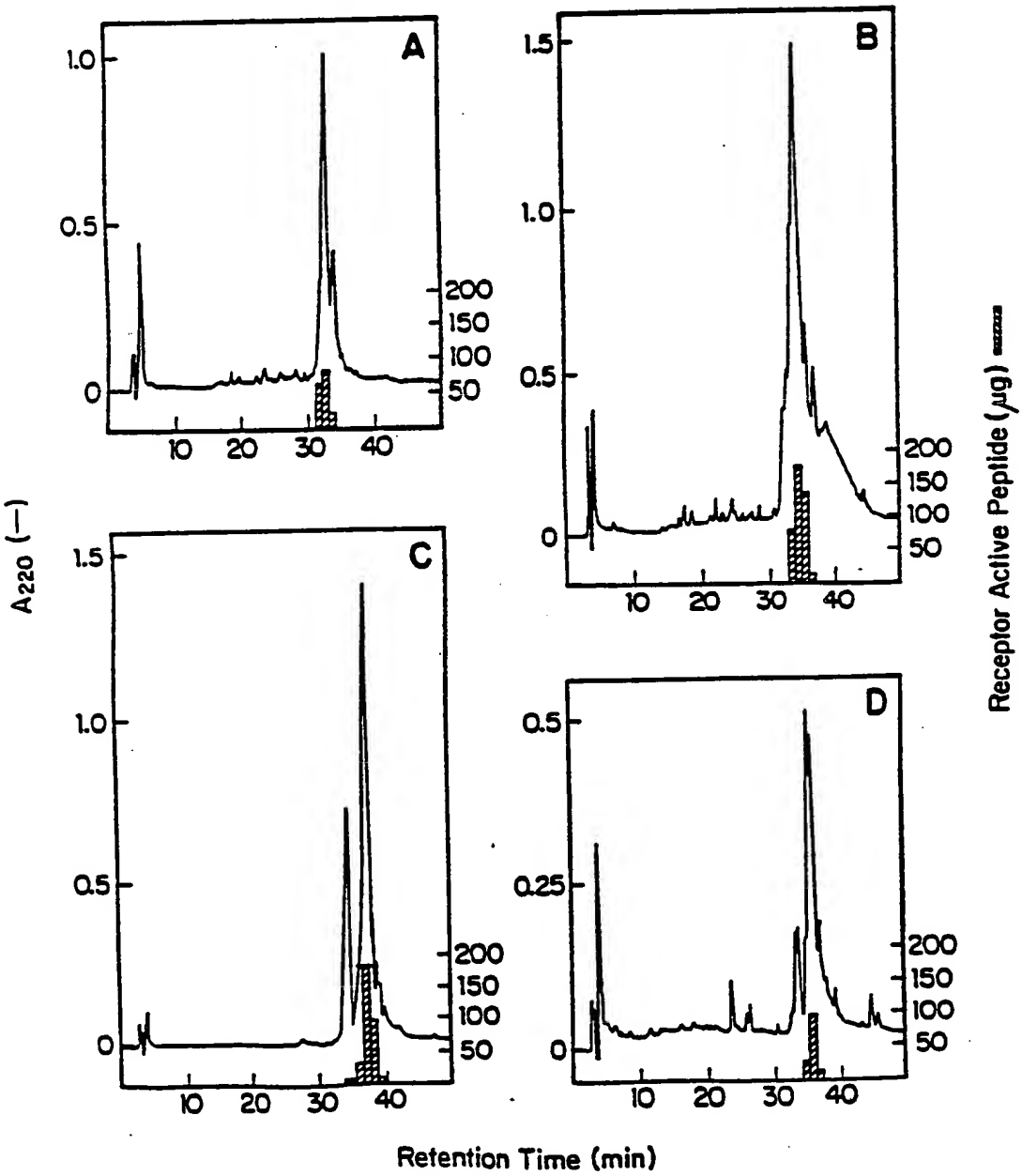


FIG.5

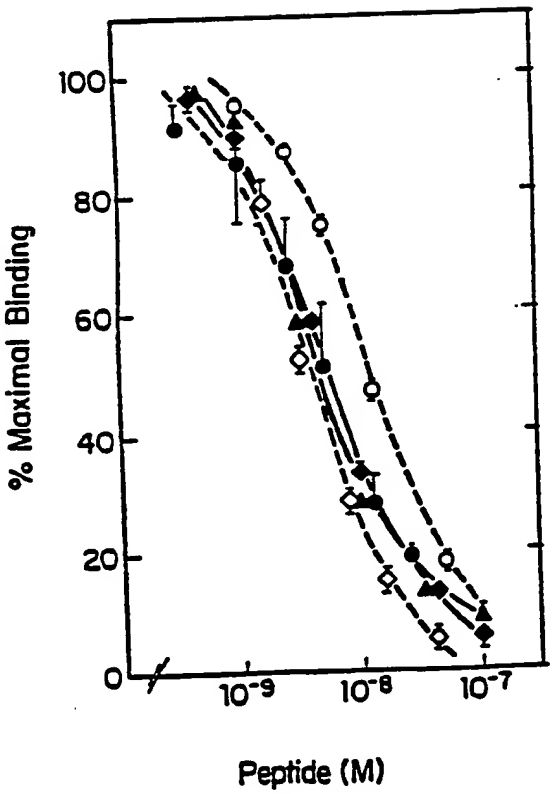


FIG.6

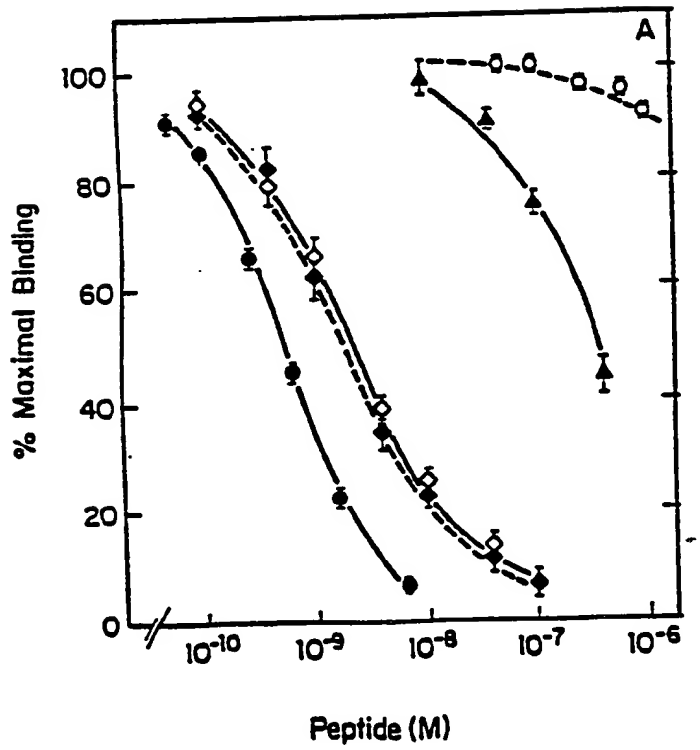
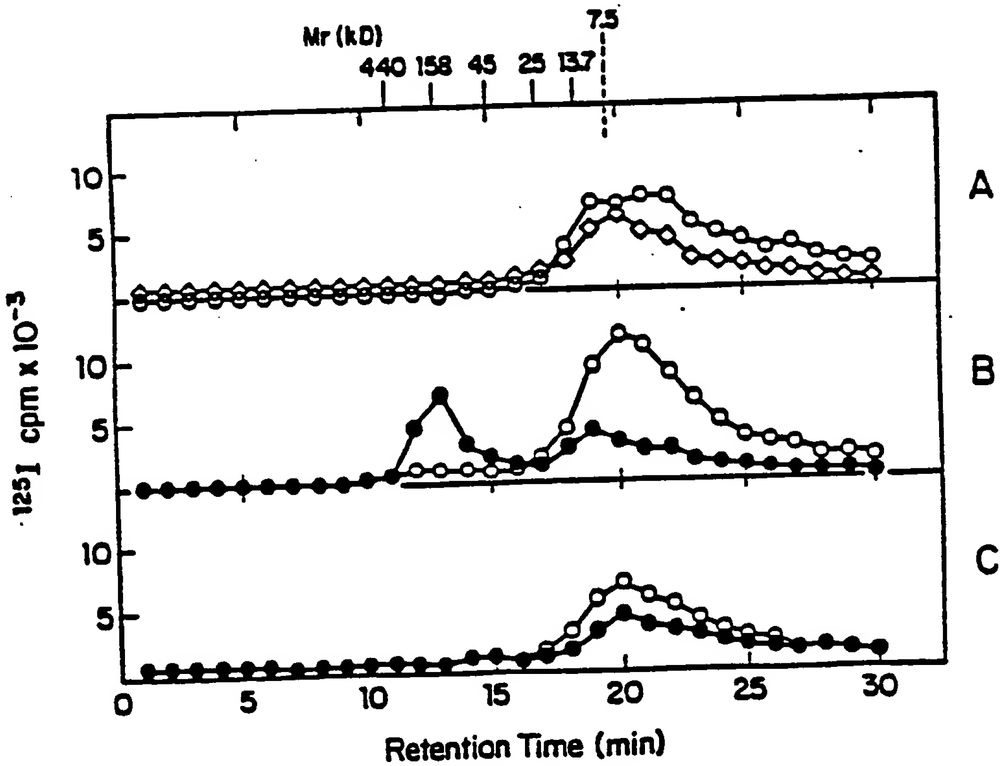


FIG.7

^{125}I-αIGF and ^{125}I-αIGF 132 Binding to Rat Serum-Analyzed by TSK 3000



A. ^{125}I-Peptides, no incubation
B. ^{125}I-αIGF + Serum ±1μM αIGF
C. ^{125}I-αIGF 132 + Serum ± 1μM αIGF 132

FIG.8

Stimulation of DNA Synthesis in Mouse 3T3 Cells by IGF I (●), IGF 132 (■-■) and IGF 252 (■--■)

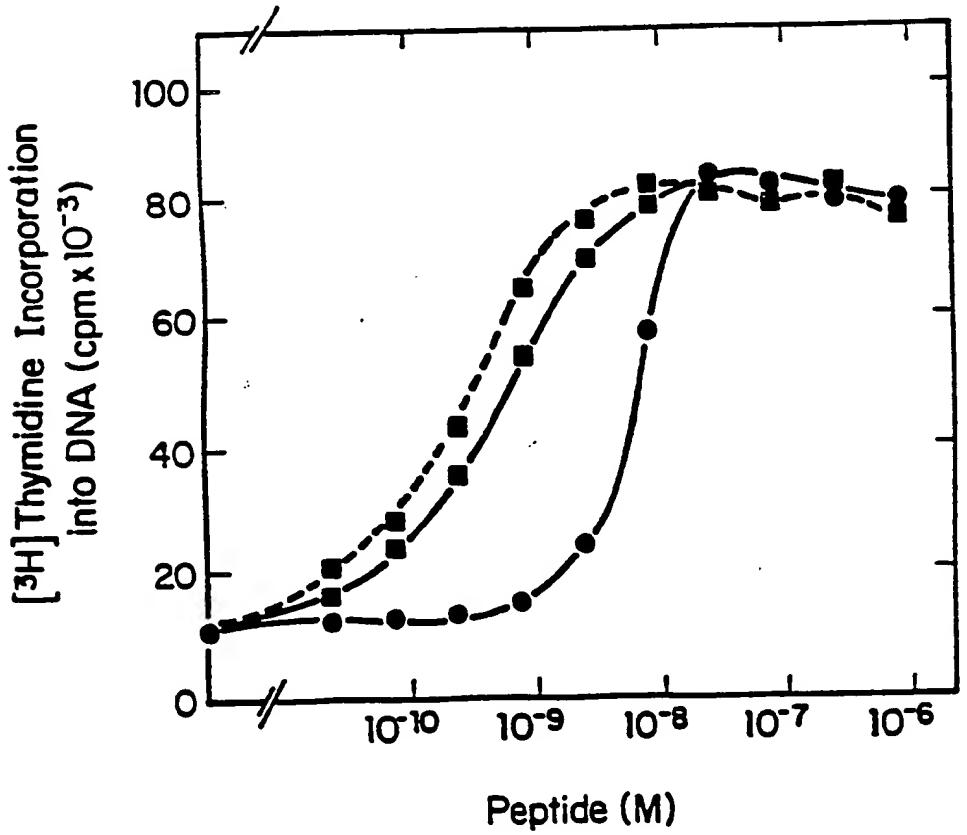


FIG. 9

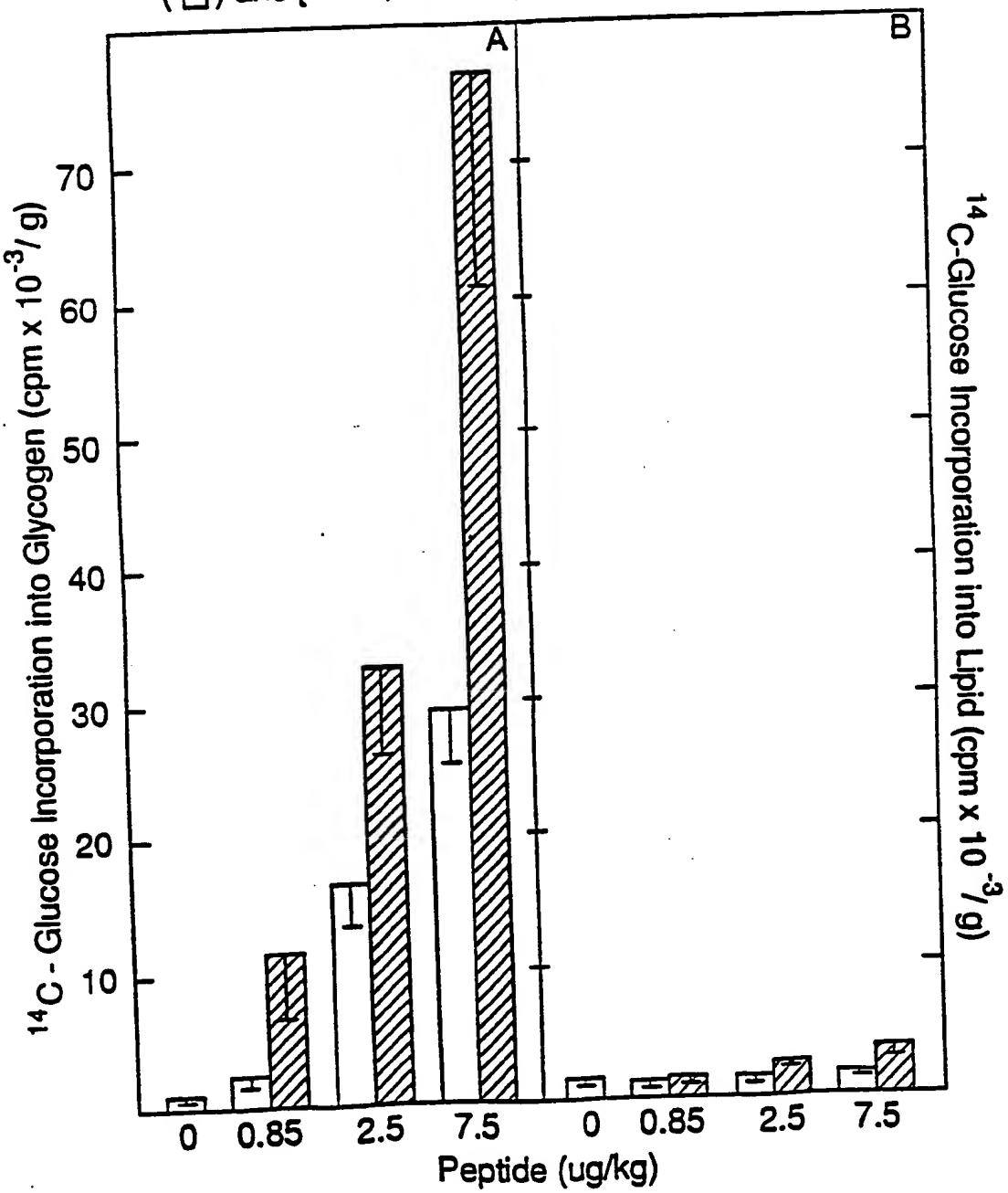


FIG. 10

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EUROPEAN SEARCH REPORT

Application Number

EP 88 20 2032

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
P,X	BIOCHEMISTRY volume 27, no. 9, 3rd May 1988, Washington, DC, USA, pages 3229-3233; M.A. CASCIERI et al.: "Mutants of human insulin-like growth factor I with reduced affinity for the tpye 1 insulin-like growth factor receptor" * abstract; page 3233, column 1; figures 1,2; table I * ---	1,8,17, 18	C 12 N 15/00 A 61 K 37/00
P,X	CHEMICAL ABSTRACTS volume 109, no. 3, 18th July 1988, page 68, abstract no. 17150r; M.L. BAYNE et al.: "Structural analogs of human insulin-like growth factor I with reduced affinity for serum binding proteins and the type 2 insulin-like growth factor receptor"; & J. BIOL. CHEM. 1988, 263(13), 6233-9 ---	1-18	
D,Y	PROC. NATL. ACAD. SCI. USA volume 82, May 1985, Washington DC, US, pages 3010-3014; M.A. DE VROEDE et al.: "Hybrid molecules containing the B-domain of insulin-like growth factor I are recognized by carrier proteins of the growth factor" * abstract; pages 3010, column 1; page 3012, column 1; page 3014, column 1 * ---	1,8,17, 18	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 N 15/00 A 61 K 37/00
A	WO-A-8 605 810 (BIOGEN NV) * page 2, line 27 - page 3, line 8; page 4, line 22 - page 5, line 27; page 8, lines 17-20; page 18, lines 18-26; claims 1,3,6-12 * --- -/-	1,8,17, 18,21, 22,25, 26	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	09-11-1988	JULIA P.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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EUROPEAN SEARCH REPORT

Application Number

EP 88 20 2032

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	EP-A-0 229 750 (WASHINGTON UNIVERSITY) * abstract; page 12, lines 1-25; page 29, line 6 - page 30, line 21; claims 27-30 *	1,8,17-26	
D,A	BIOCHEMISTRY volume 24, February 1985, Washington, DC, US, pages 4208-4212; S. JOSHI et al.: "Synthesis of an insulin-like compound consisting of the A chain of insulin and a B chain corresponding to the B domain of human insulin-like growth factor I" * abstract; page 4212; figure 6 and discussion *	1,8,17, 18	
Y	WO-A-8 500 831 (AMGEN) * abstract; page 4, lines 2-4; page 5, line 21 - page 6, line 15; claims 1,2,13,18,24,26-35 *	1,8,17, 18	
A		21-22, 25-26	TECHNICAL FIELDS SEARCHED (Int. Cl.4)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	09-11-1988	JULIA P.

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A : technological background
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P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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